Metabolically Engineered Micro-Organisms Having Improved Galactose Uptake

Background of the Invention

5   Galactose is an abundant sugar in many different raw materials for industrial
fermentation processes. Thus, galactose is one of the sugars present in lactose, the
primary sugar of whey. It is also present in raffinose, a sugar present in beet molasses.
Furthermore, it represents 3-18% of the sugars present in hemicellulose from various
plant sources. Galactose can be metabolized by most micro-organisms. However, the
10   rate of uptake of galactose is for most organisms substantially lower than the uptake
of the sugars glucose, fructose and mannose. For exploitation of galactose as a raw
material for industrial fermentations in the production of low value added products
like ethanol, lactic acid and citric acid the slow uptake rate of galactose represents a
fundamental problem.

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Within the last decade metabolic engineering has been successfully applied for
optimisation of several industrial fermentation processes (Nielsen, 2001).
Recombinant DNA techniques have facilitated the ability to genetically modify
suitable host systems, and this has resulted in recombinant strains that have reduced
20   by-product formation with a resulting increase in the overall yield of product. For
many industrial processes - especially those involving low-value added products - the
yield is certainly important, but it is also important to have a high rate of conversion
of the substrate into the product. This requires optimisation of the flux through the
central carbon metabolism, which has been attempted for glycolysis in
25   *Saccharomyces cerevisiae* (Schaff *et al.*, 1989) and in other micro-organisms. These
attempts have, however, largely failed for two major reasons. First, control of flux
through central carbon metabolism is often distributed over many enzymes, so an
increase in flux requires increased activity of many (or all) enzymes in the pathway.
Second, regulation of glycolysis - both at the genetic and at the enzymatic level - is
30   generally believed to be very rigid making it difficult to modulate flux through
amplification of individual enzyme activities.

One way to solve these problems may be over expression of many (or all) genes encoding the enzymes in a given pathway. However, this may impose a physiological burden on the cell by draining pools of nucleotides or amino acids, or by slowing down transcriptional or translational efficiency. This may have metabolic

5   consequences in other parts of metabolism, which negatively affects the overall performance of the cell. Furthermore, high levels of all the enzymes in the pathway may lead to significant changes in metabolite levels, which may result in down-regulation of some enzymes.

10   In fungi the uptake and metabolism of galactose is via the Leloir pathway (see Figure 1). In this pathway galactose is first transported into the cell by a specific permease. In the next step the sugar is phosphorylated into galactose-1-phosphate, a reaction that is catalyzed by a specific kinase. In the next step of the pathway galactose-1-phosphate reacts with UDP-glucose and forms glucose-1-phosphate and UDP-galactose, a

15   reaction that is catalyzed by galactose-1-phosphate uridylyltransferase. Regeneration of UDP-glucose from UDP-galactose is catalyzed by a separate enzyme involved in the pathway – UDP-glucose 4-epimerase. Glucose-1-phosphate is the end product of the Leloir pathway, but in the further catabolism of galactose this sugar phosphate is further converted into glucose-6-phosphate, which may be further processed via the

20   Embden Meyerhof Parnas pathway or the Pentose Phosphate pathway. The conversion of glucose-1 phosphate to glucose-6 phosphate is catalyzed by the enzyme phosphoglucomutase (PGM). As glucose-1 phosphate is used as precursor for trehalose, glycogen and glucan biosynthesis, PGM also plays a role during metabolism of glucose, but here it catalyzes the conversion of glucose-6 phosphate to

25   glucose-1 phosphate.

The galactose metabolism is subjected to dual control, being induced by galactose and repressed by glucose. Regulation has been extensively studied in the yeast *Saccharomyces cerevisae*, which preferably uses glucose as energy and carbon source

30   over galactose. In this yeast the genes involved in galactose metabolism, often referred to as the *GAL* genes, are subjected to glucose repression to a much larger extent than other genes controlled by glucose such as the *MAL* genes and the *SUC* genes (Johnston and Carlson, 1992; Klein et al., 1998). The structural *GAL* genes subjected to this dual control are the *GAL2* gene, encoding galactose permease, the

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GAL1 gene, encoding galactokinase, the *GAL7* gene, encoding galactose-1-phosphate uridylyltransferase, and the *GAL10* gene, encoding UDP-glucose 4-epimerase. Transcription of these structural *GAL* genes is enhanced 1000-fold after de-repression by glucose and induction by galactose (Melcher, 1997). In *S. cerevisiae*

5   phosphoglucomutase is often referred to as the Gal5 protein, but as there are two isoforms of the enzyme a more correct description of this enzyme is designation of each isoform encoded by the two genes *PGM1* and *PGM2*. Both these genes are not under the same tight control as *GAL1, GAL2, GAL7* and *GAL10*, as their transcription are only increased threefold in the presence of galactose (Oh and Hopper, 1990). This

10   is likely a consequence of the high basic level of expression of the *PGM2* gene at non-induced conditions independent of the positive transctiptional activator Gal4 (Oh and Hopper, 1990), which may be due to the role of phosphoglucomutase in the glycogen, trehalose and glucan biosynthesis. In fact phosphoglucomutase is generally believed to be in excess as it can equilibrate the pools of glucose-1 phosphate and glucose-6

15   phosphate (Zubay, 1988). Furthermore this enzyme has a high affinity for its substrates (Daugherty et al. 1975). For these reasons gene products of *PGM1* and *PGM2* are generally not believed to exert any degree of flux control in the Leloir pathway, which is consistent with the situation of the glycolysis where no single enzyme has been shown to exert any significant flux control.

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The system involved in regulation of the expression of the structural *GAL* genes is illustrated in Figure 2. The regulatory *GAL* gene products comprise Gal3 and Gal4 that are necessary for induction of the *GAL* genes. The protein Gal4 acts as a transcriptional activator of the *GAL* genes by binding to specific sequences upstream

25   of the coding region (Johnston, 1987). The *GAL80* gene encodes a protein that binds to Gal4 and prevents this protein from activating transcription. Recent in vitro studies strongly indicated that Gal3 interacts with Gal80 and the interaction of Gal3 with Gal80 is believed to relieve the interaction between Gal80 and Gal4 which covers the activating domain of Gal4, and hence, transcriptional activation of the *GAL* genes is

30   possible (Platt and Reece, 1998). Another regulatory gene recently devoted to the group of *GAL* genes is the *GAL6* gene, which has been shown to be regulated by galactose (Zheng *et al.*, 1997). *GAL6* is regulated in similar way as *GAL80* and the gene product has a negative impact on expression of the GAL genes, but the actual mechanism of action within the *GAL* system still remains to be elucidated. Finally,

Mig1-mediated glucose repression also imposes control over the *GAL* system (Johnston and Carlson, 1992). The Mig1 protein takes part in a protein complex with the proteins Ssn6 and Tup1, which binds to the *GAL1* promoter and the *GAL4* promoter (Keleher *et al.*, 1992; Treitel and Carlson, 1995; Frovola et al., 1999) and

5   hence preventing transcription of these two genes. Repression of the latter gene has a major effect by down-regulation of the whole *GAL* system.

Ostergaard *et al.* (2000) report on modulation of the regulatory *GAL* system for improving the uptake and metabolism of galactose. They constructed and analysed a

10   number of recombinant strains with alteration in the expression of regulatory genes. The best effect was obtained by deleting the three genes *MIG1*, *GAL80* and *GAL6* whereby the galactose uptake rate increases 41%. A positive effect was also found by over expression of *GAL4* resulting in a 26% increase in the galactose uptake rate. Deletion of *GAL6* alone results in a 24% increase in the galactose uptake rate,

15   whereas deletion of *MIG1* and *GAL80* alone increases the galactose utilisation with 15% (Ostergaard *et al.*, 2000). Based on these findings it was speculated that deletion of negative regulatory genes like *MIG1*, *GAL80* and *GAL6* and over expression of the gene *GAL4* encoding the positive transcriptional regulator results in an co-ordinated and balanced increased expression of the structural *GAL* genes *GAL1*, *GAL2*, *GAL7*

20   and *GAL10* leading to an increased flux through the Leloir pathway.

Masuda *et al* (2001) investigated the effect of lithium on the metabolism of galactose in *Saccharomyces cerevisiae* and in particular on the phosphoglucomutase activity due to transcription of the PGM2 gene. One of the constructs they used was an

25   auxotrophic (leucine and histidine requiring) strain over expressing *PGM2*. Such a strain would be unsuitable for commercial use because of its auxotrophic nature and would not be able to grow on minimal media (i.e. media not supplemented to supply the amino acids required by the auxotrophic strain. This strain is used in Masuda *et al.* only as a basis for comparison in the investigation of the effect of lithium with a

30   view to better understanding the mechanism of the therapeutic effect of lithium in treating manic depressive disorder in humans. *PGM2* over expression was used only to demonstrate that this could overcome the suppression of *PGM2* activity otherwise produced by lithium stress. No beneficial result relevant to the normal cultivation of *S. cerevisiae* was reported.

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In the present invention we applied genome-wide transcription analysis using DNA arrays to identify differently expressed genes in three of the strains with different galactose utilisation capacity studied by Ostergaard *et al.* (2000). Based on this

5   analysis we identified PGM2 to be an important gene for ensuring a high galactose uptake rate.

Brief summary of the invention

10 The present invention now provides a recombinant, prototrophic micro-organism exhibiting an increased level of galactose uptake rate when cultured on a nutrient source providing galactose, said micro-organism being a yeast or other fungi having the ability to grow on minimal medium and over expressing the activity of an enzyme catalysing the conversion of glucose-1 phosphate to glucose-6 phosphate in the

15 galactose uptake and metabolism pathway compared to a reference micro-organism having a native level of activity of said enzyme and from which the recombinant micro-organism is derived.

Other genetic modifications of a starting non-recombinant strain may be made before,

20 after or simultaneously with the introduction of the modification giving rise to over-expression of the said enzyme activity.

Detailed description of the invention

25 Said micro-organism may be a fungi and said enzyme may be a phosphoclugomutase. Specifically the enzyme may be Gal5 and may be encoded by either or both of the genes *PGM1* and *PGM2*.

Preferably, said enzyme activity is expressed in the micro-organism at a level which is

30 at least 1.5 times that of the said reference micro-organism.

The over expression may be brought about by introducing multiple copies of a gene coding for the said enzyme. Alternatively or additionally, a gene coding for the said enzyme may be under the control of a genetic control sequence which has been

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recombinantly introduced and which is not natively associated with said gene, leading to said over expression of said enzyme activity. Alternatively or additionally there may be present a gene coding for a mutated form of the said enzyme which mutated form has a higher specific activity than the native form of said enzyme of said micro-

5   organism. Specific methodologies for mutating yeast strains to produce over expression of genes by all of these methods are well known in the art.

Preferably, the micro-organism is a yeast. It may be a strain of *Saccharomyces cerevisiae*. More generally the micro-organism may be a species belonging to the

10   genus *Saccharomyces*, e.g. *S. cerevisiae*, *S. kluyveri*, *S. bayanus*, *S. exiguus*, *S. sevazzi*, *S. uvarum* , a species belonging to the genus *Klyuveromyces*, e.g. *K. lactis K. marxianus var. marxianus*, *K. thermotolerans*, a species belonging to the genus *Candida*, e.g. *C. utilis C. tropicalis*, a species belonging to the genus *Pichia*, e.g. *P. stipidis*, *P. pastoris*, *P. sorbitophila*, or other yeast species, e.g. *Debaromyces*

15   *hansenii*, *Hansenula polymorpha*, *Yarrowia lipolytica*, *Zygosaccharomyces rouxii* or *Schizosaccharomyces pombe*.

Concerning other fungal micro-organisms, a non-exhaustive list of suitable micro-organisms will include the following:

20   *Aspergillus niger, Aspergillus awamori, Aspergillus oryzae, Aspergillus nidulans, Penicillium chrysogenum, Rhizopus oryzae.*

The micro-organism preferably exhibits an increase of the maximum specific

25   galactose uptake rate of at least 10%, more preferably at least 20%, for instance at least 40%, in comparison to a said reference micro-organism.

Preferably, the micro-organism exhibits an increase of said enzyme activity of at least 2 fold, more preferably at least 5 fold, more preferably at least 10 fold, more

30   preferably at least 20 fold, more preferably 100 fold, in comparison to a said reference micro-organism.

Preferably, the micro-organism exhibits an increased maximum specific ethanol production rate compared to said reference micro-organism, e.g. said ethanol

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production is increased by at least a factor of 1.1, more preferably by at least a factor of 1.5.

Brief description of the accompanying drawings

Figure 1 shows an overview of the Leloir pathway.

Figure 2 shows the regulation of the *GAL* system in *S. cerevisiae*.

Figure 3 shows the result of batch cultivation of the strain SO7 in Example 2 below.

Figure 4 shows the result of batch cultivation of the *PGM2* strain in Example 6 below.

As described below, we have genetically engineered a strain of *S. cerevisiae* to over express the *PGM2* gene by transformation with a multicopy plasmid vector containing the *PGM2* gene under the control of a strong promoter to obtain a non-auxotrophic (prototrophic) strain exhibiting increased PGM enzyme activity whilst being able to grow on minimal medium (and specifically being able to grow in the absence of added leucine and histidine). It is found that surprisingly this strain has increased flux through the galactose pathway when cultured with a supply of galactose, leading to increased uptake of galactose and increased ethanol production.

The invention will be further described with reference to the following Examples which are non-limiting and presented for illustration.

Example 1 Construction of yeast strains

All *S. cerevisiae* strains used were generated from the CEN.PK113-7D (*MAT*a) wild type strain (van Dijken et al. 2000). SO7 containing the 2μ high-copy vector pBM959 with *GAL4* behind its native promoter and *URA3* as marker and SO16 (*gal80*, *mig1*, *gal6*) were constructed by Ostergaard and co-workers (Ostergaard *et al.*, 2000). The *PGM2* strain overexpressing *PGM2* was constructed by transforming CEN.PK113-5D

(ura3) with the 2µ high-copy vector p*PGM2* (Masuda et al., 2001) containing *PGM2* behind the *PMA1* gene promoter and *URA3*. Strains were stored at -80°C in 20% (v/v) glycerol. These frozen stocks were used for obtaining single colonies on plates with a selective minimal medium for inoculation of precultures.

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Example 2 Batch cultivations of WT, SO7 and SO16

The *S. cerevisiae* strains either overexpressing *GAL4* (SO7) or deleted in *GAL6*, *GAL80* and *MIG1* (SO16) and the corresponding reference strain CEN.PK113-7D (WT) were examined under controlled conditions in aerobic batch cultivations on a

10   minimal media with a start concentration of 15 g/l galactose. All the strains were prototrophic thereby avoiding addition of various amino acids that could affect transcription of amino acid related genes. Cultivations of all strain were done in triplicates and samples for DNA arrays were harvested at a residual galactose concentration of 7.5 ±2.0 g/l.

15   The batch cultivations were carried out in well-controlled laboratory fermenters with a working volume of 2 or 4 litres. A defined medium (Verduyn *et al.*, 1992) was used, which contained per litre: 15 g galactose; 5.0 g $(NH_4)_2SO_4$; 3.0 g KH_2PO_4; 0.5 g $MgSO_4$, $7H_2O$; and trace metals and vitamins as described by Verduyn *et al.* (1992). 50 µl/L antifoam (Sigma A-8436) was added to avoid foaming. Galactose was

20   autoclaved separately from the mineral medium and afterwards added to the fermenter together with a sterile filtrated solution containing the vitamins.

Precultures were grown at 30°C and 150 rpm in cotton-stopped, 500 ml Erlenmeyer flasks with baffles containing each 100 ml of a media of pH 6.5 similar to that in the fermenters, but containing different concentrations of galactose (20 g/L), $(NH_4)_2SO_4$

25   (7.5 g/L) and KH_2PO_4 (14 g/L). Precultures were inoculated from plate cultures, which were inoculated directly from frozen stocks, and grown at 30°C on selective media. Exponential phase precultures were used to inoculate to a start concentration of 1 mg CDW/L.

Cultivations were carried out at 30°C with a stirrer speed of 800 rpm and were aerated

30   with air at a flow rate of 1 litre per litre staring volume per minute. The concentration of dissolved oxygen was measured with a Mettler Toledo polarographic electrode and remained above 70%. pH was kept at 5.0 by automatic addition of 4 M KOH. The

bioreactors were fitted with cooled condensers, and the off-gas was led to a gas analyser (INNOVA, Denmark) to measure the content of CO_2.

The concentration of biomass in batch fermentations was determined on a dry weight basis by filtering a known volume of culture through a pre-weighed 0.45 μm nitrocellulose filter (Gelman Sciences, Ann Arbor, Mich.). The filter was washed with distilled water, dried in a microwave oven at 150 W for 15 minutes and finally weighed to determine its increase in dry weight.

Culture samples for determination of galactose, ethanol, glycerol, acetate, pyruvate and succinate concentrations were filtered through a 0.45 μm cellulose acetate filter (Osmonics) immediately after sampling, and the filtrate was frozen at -20°C until further analysis. The concentrations of the metabolites were determined by high-pressure liquid chromatography on an Aminex HPX-87Hm column (Bio-Rad) kept at 65°C and eluted at 0.6 ml per minute with 5 mM H_2SO_4. Acetate and pyruvate were detected spectrophotometrically by a Waters 486 Turnable Absobance Detector at 210 nm. Galactose, ethanol, glycerol and succinate were detected refractometrically by a Waters 410 Differential Refractometer.

Results from the batch cultivation with the strain SO7 are shown in Figure 3 and overall results from batch cultivations of all three strains are shown in the table below.

	WT	SO7	SO16
Specific growth rate (h^{-1})	0.17 ±0.01	0.18 ±0.01	0.18 ±0.00
Specific galactose uptake rate (g galactose/g DW/h)	0.60 ±0.03	0.80 ±0.04	0.84 ±0.02
Specific ethanol production rate (g ethanol/g DW/h)	0.11 ±0.01	0.19 ±0.02	0.22 ±0.01
Yield of biomass on galactose (g DW/g galactose)	0.22 ±0.01	0.22 ±0.01	0.22 ±0.01

Data is given as the average of three similar independent cultivations ± the standard deviation.

Example 3 Genome-wide transcription analysis

Oligonucleotide microarrays from Affymetrix containing sequences for all know ORFs in *S. cerevisiae* were used for genome-wide expression analysis during batch cultivations with the strains SO7, SO16 and WT.

To find genes, which had changed expression in at least one of the two mutants an ANOVA analysis was performed on the 5963 probe sets representing ORFs in *S. cerevisiae* that were called present on at least one array. Only 24 ORFs showed a significantly changed expression in at least one of the two mutants when using a global likelihood of getting 1 false positive (cutoff at $P=1.68 \cdot 10^{-4}$), and most of these ORFs had only changed expression in the *GAL4* overexpression strain. The 30 best scoring genes in the ANOVA test are shown in the table below.

ORF		Function	Fold change[a]		P-value[b]
			SO7	SO16	
YGL157W		Unknown	3.7	-1.5	3.53E-08
YEL021W	*URA3*	Pyrimidine biosynthesis	5.2	-1.2	5.66E-07
YPL248C	*GAL4*	Galactose regulation	23	1.3	5.95E-07
YBR298C	*MAL31*	Maltose metabolism	-1.4	1.1	3.85E-06
YML051W	*GAL80*	Galactose regulation	1.3	-2.1	7.90E-06
YJL056C	*ZAP1*	Transcriptional regulation	2.3	1.1	1.31E-05
YLR035C	*MLH2*	DNA repair	3.1	-1.5	2.60E-05
YBR093C	*PHO5*	Phosphate metabolism	32	1.0	3.11E-05
YIL057C		Unknown	-1.2	1.8	3.77E-05
YGL130W	*CEG1*	mRNA capping	2.1	1.0	5.54E-05
YOL143C	*RIB4*	Riboflavin biosynthesis	1.7	1.2	5.76E-05
YKL031W	.	Unknown	9.4	1.9	6.04E-05
YLR286C	*CTS1*	Cell wall biogenesis	-1.4	1.1	7.30E-05
YNL239W	*GAL6*	Galactose rebgulation	2.0	-150	7.65E-05
YGR022C		Unknown	10	1.0	9.68E-05
YEL069C	*HXT13*				
YDL245C	*HXT15*				
YJR158W	*HXT16*				
YNR072W	*HXT17*	Hexose transporters	1.0	3.2	9.95E-05
YBR299W	*MAL32*				
YGR292W	*MAL12*	Maltose metabolism	-2.3	1.2	1.07E-04
YOL126C	*MDH2*	Gluconeogenesis	2.1	-1.2	1.13E-04
YFL011W	*HXT10*	Hexose transporter	2.7	1.7	1.19E-04
YMR305C	*SCW10*	Mating	-1.6	-1.2	1.37E-04

YKL216W	URA1	Pyrimidine biosynthesis	1.7	1.1	1.42E-04
YDR520C		Unknown	1.7	1.1	1.42E-04
YGL035C	MIG1	Glucose repression	-1.3	-17	1.46E-04
YPL187W	MFα1	Mating	-2.1	-2.1	1.48E-04
YMR199W	CLN1	Cell cycle	-1.7	-1.1	1.63E-04
YJR159W	SOR1	Fructose metabolism			
YDL246C		Unknown	2.2	1.4	1.83E-04
YMR176W	ECM5	Cell wall biogenesis	2.1	1.1	2.04E-04
YOL058W	ARG1	Arginine biosynthesis	1.2	1.0	2.19E-04
YOR378W		Unknown	5.8	1.5	2.49E-04
YGR249W	MGA1	Filamentous growth	-3.2	-2.4	2.53E-04

[a] Fold change compared to wild type strain.

[b] P-value returned by ANOVA. The lower the value the higher probability of a significant changed expression.

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Five of the 24 ORF with changed expression are *GAL4*, *GAL6*, *GAL80*, *MIG1* and *URA3*, which were deleted or over-expressed in one of the two mutants. Of these five ORFs *GAL4*, *GAL6* and *GAL80* showed possible changes in the strain were they had not been deleted or over-expressed – a 1.3-, 2.0- and 1.3-fold up-regulation

10 respectively. We expected to see many of the Gal4 activated genes to be up-regulated, but *GAL6* is the only gene with a Gal4 binding site in its promoter, which was up-regulated among the 24 significantly changed genes. The hypothetical ORF YIL057C also has a putative Gal4 binding site in its promoter (Ideker *et al.*, 2001), but is down-regulated 2.2 fold in SO7. From the significantly changed genes we could not identify

15 any genes with changed expression in both mutants, which could be new candidates as key enhancers of the flux through the galactose utilisation pathway. Most of the genes with significantly changed expression are likely to be secondary effects of over-expressing *GAL4*, since many of them has changed expression in SO7 only.

20 *HXT10*, which is able to transport galactose (Wieczorke et al. 1999) is up-regulated in both SO7 and SO16 and could be a possible key enhancer, but *HXT10* is lowly expressed compared to *GAL2*. The probe set representing *HXT13*, *HXT15-17* was up-regulated 3.2 fold in SO16, however, none of these *HXT*'s are able to transport galactose (Wieczorke *et al.* 1999). The hypothetical ORF YKL031W is up-regulated

9.4 fold in SO7 and 1.9 fold in SO16 compared to the wild type strain. YKL031W is coding a protein of 137 amino acids which binds phosphoinositol 4,5-biphosphate *in vitro* (Zhu et al., 2001) and contains two predicted transmembrane segments (Purnelle and Skala, 1994), but which has no related proteins.

Surprisingly expression of *GAL1*, *GAL2*, *GAL7* and *GAL10* were not higher in the mutants than the wild type. Apparently they were already expressed at their maximum level in the wild type strain.

Example 4 Identification of target for metabolic engineering

The genome-wide transcription analysis of the two mutants did not identify any new gene candidates for being key enhancers of the galactose flux. Nor did it indicate any reason for the mutant's increased galactose uptake rates, such as significantly increased transcription levels of the genes encoding the Gal enzymes. We therefore looked further into the transcription results for the *GAL* genes, which are the most obvious candidates for key enhancers, and which we had hypothesised would be up-regulated to balanced levels in the two mutants. Results of this analysis are shown in the table below.

| | Transcription | | |
| | Fold change[a] | | |
Gene	SO7	SO16	P[b]
GAL4	23	1.3	$6.0 \cdot 10^{-7}$
GAL80	1.3	-2.1	$7.9 \cdot 10^{-6}$
GAL6	2.0	< -100	7.710^{-5}
PGM2	1.7	1.3	$6.1 \cdot 10^{-3}$
GAL7	1.0	1.1	0.17
GAL3	1.7	1.0	0.18
GAL2	1.0	1.1	0.38
GAL10	1.0	1.1	0.46
PGM1	1.2	1.1	0.57
GAL1	1.0	1.0	0.99

a Fold change compared to wild type strain.

b P-value returned by ANOVA. The lower the value the higher probability of a significant changed expression.

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Except from *GAL4*, *GAL6*, and *GAL80*, only *PGM2*, encoding the major isoform of phosphoglucomutase, Gal5, had a high probability of being significantly changed. For the genes encoding the first four enzymes in the galactose utilisation pathway the probability of a changed expression was less than 83 % when not even considering

10 multiple testing. Taking multiple testing of the 10 *GAL* genes into consideration there was a 94% probability of a changed expression of *PGM2* in at least one of the two mutant strains. *PGM2* expression had only increased 1.7 and 1.3 fold in SO7 and SO16 respectively, so

15 On the basis of the transcription analysis only *PGM2* was identified as a possible new key enhancer gene for increasing the galactose flux.

Example 5 Measurement of enzyme activities

To verify that concentration of active Gal5 had indeed a higher increased expression

20 and that other Gal enzymes had not, the *in vitro* enzyme activities of Gal1, Gal7 and Gal5 were measured in cell free extracts from the same cultivations.

Cell free extracts were produced with the help of a Fastprep FP120 instrument (Savant Instruments, New York) as previously described (Møller *et al.*, 2002). *In vitro* enzyme

25 activities of phosphoglucomutase (Pgm) and galactose-1-phosphate uridyl transferase (Gal7) were assayed at 30°C by following the NADPH production at 340 nm using a spectrophotometer (HP 8353 UV-VIS system with Chemstation software from Hewlett Packard). Pgm and Gal7 activities were determined as earlier described (Bergmeyer *et al.*, 1983; Elsevier *et al.*, 1996) respectively, in a 1 ml reaction mixture

30 activity at a minimum of two different concentrations of cell free extract. Galactokinase (Gal1) activity was measured with a method modified from (Mizoguchi *et al.*, 1993). The reaction mixture contained: 0.1 M phosphate buffer (pH 7.5); 3.0 mM $MgCl_2$; 1.8 mM NaF; 1.5 mM ATP and four different concentrations of cell free

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extract. After 5 minutes at 30°C adding galactose to 2.5 mM started the reaction, and the reaction mixture was incubated for another 5 minutes and then boiled for 2 minutes. The amount of consumed galactose was determined by measuring the residual concentration of galactose enzymatically (Boehringer Mannheim kit 176303) after centrifugation (10 minutes, 1000g). Protein content in cell free extracts was determined by the Lowry method (Lowry *et al.*, 1951), using fatty-acid free BSA (Sigma A-6003) as standard.

The results of the *in vitro* enzyme activity measurements supported the conclusion from the transcription analysis. The results are shown in the table below.

| | *In vitro* Enzyme activity | |
| | Fold change[a] | |
Enzyme	SO7	SO16
Gal7	1.6	-1.3
Gal10	-1.2	1.2
Gal5	2.6[b]	1.5[b]

[a] Fold change compared to wild type strain.

[b] Fold change of total phosphoglucomutase activity.

The *in vitro* enzyme activities of Gal1 and Ga7 did not change significantly in any of the two mutants compared with the reference strain, while the *in vitro* activity of Gal5 had increased 2.6 and 1.5 in SO7 and SO16 respectively.

Example 6 Over expression of *PGM2*

To test the new hypothesis that Gal5 does exert control over the flux through the galactose utilisation pathway we constructed a strain over expressing *PGM2*. *S. cerevisiae* was transformed with a multicopy expression vector from Masuda *et al.* (2001) containing *PGM2* under the control of the strong *PMA1* gene promoter.

The *S. cerevisiae* strain with over expression of *PGM2* was compared with the corresponding reference strain CEN.PK113-7D (WT) under controlled conditions in

aerobic batch cultivations on a minimal media with a start concentration of 15 g/l galactose. Cultivations of both strains were done in triplicates. The batch cultivations were carried out as described in Example 2.

5   Results from the batch cultivation with the *PGM2* strain are shown in Figure 4 and overall results from batch cultivations of the strain and the WT are shown in the table below. Data is given as the average of three similar independent cultivations ± the standard deviation.

	WT	*PGM2* strain
Specific growth rate (h^{-1})	0.17 ±0.01	0.23 ±0,02
Specific galactose uptake rate (g galactose/g DW/h)	0.60 ±0.03	1.04 ±0,07
Specific ethanol production rate (g ethanol/g DW/h)	0.11 ±0.01	0.30 ±0,03
Yield of biomass on galactose (g DW/g galactose)	0.22 ±0.01	0.22 ±0,01

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The resulting *PGM2* strain had a significantly increased maximum specific galactose uptake rate compared to the both the reference strain and the two other mutants. Overexpressing *PGM2* resulted in a 70% increase in the maximum specific galactose uptake rate and a 3 times higher maximum specific ethanol production rate compared

15   to the wild type strain showing that Gal5 is rate controlling for galactose utilisation. The maximum specific growth rate of the *PGM2* strain was not negatively affected, but rather increased. *In vitro* enzyme activity measurements verified that the concentration of active phosphoglucomutase had indeed increased in the *PGM2* strain, showing 17-fold higher phosphoglucomutase activity. However, the *in vitro* enzyme

20   activity of Gal7 had also increased with approximately 3 fold in the *PGM2* strain. This might have a positive effect on the galactose utilisation rate as well.

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